BIOCHEMICAL STUDIES OF ω-CONOTOXIN GVIA; A PEPTIDE TOXIN INHIBITING VOLTAGE-SENSITIVE Ca^{++} CHANNELS

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INTRODUCTION

The fish-hunting cone snails use their venom to quickly paralyze their more agile prey. In the last few years, our laboratories have carried out a program of analyzing biologically active components present in the fish-hunting cone snail venoms (Cruz et al. 1985; Olivera et al. 1985). We have concentrated on the venom of one piscivorous species, Conus geographus L. which is known to have caused human fatality.

Our work has revealed that the venom of Conus geographus contains three classes of paralytic toxins (Olivera et al. 1985). The first class has physiological activity analogous to the α-neurotoxins (i.e., α-bungarotoxin) from snakes; we have named these peptide toxins, which are 13-15 amino acids long, the α-conotoxins. Their target is the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction (Gray et al. 1981). The α-conotoxins compete for binding to the AChR at the neuromuscular junction with α-bungarotoxin and curare, indicating that the binding sites in the nicotinic AChR must overlap.

A second class of paralytic toxins in the venom of Conus geographus are the μ-conotoxins, which have physiological activity analogous to tetrodotoxin and saxitoxin, except that they appear to be highly specific for muscle. Their targets are sodium channels (Spence et al. 1977; Sato et al. 1983; Cruz et al. 1985b). It has recently been shown that the μ-conotoxins, which are peptide toxins of 22 amino acids, compete with tetrodotoxin and saxitoxin for binding to muscle sodium channels, or muscle-derived sodium channels such as are found in the electric organ of the electric eel (Moczydlowski et al. 1986; Yanagawa et al. 1986; Ohizumi et al. 1986). Unlike tetrodotoxin and saxitoxin, however, they strongly discriminate against neuronal Na channels. In certain systems more than a 1,000-fold discrimination between muscle and neuronal sodium channels was observed (Cruz et al. 1985a). Thus, μ-conotoxin effects on peripheral neuronal sodium channels are probably physiologically unimportant; it is the ability

of this toxin to directly inhibit muscle action potentials which accounts for its in vivo effects.

In this paper, however, we will mainly discuss the third class of paralytic toxins that have been found in Conus geographus venom, the ω-conotoxins (Olivera et al. 1984). These peptide toxins, 25-29 amino acids long, have a novel biological activity, quite different from that of any toxin heretofore described. Their targets are voltage sensitive Ca channels. The ω-conotoxins comprise a discrete set of homologous peptides, which can be roughly divided into two classes based both on sequence and biological activity. The structures of all ω-conotoxins are shown in Figure 1; most of the biochemical and electrophysiological work that has been done has been done with ω-conotoxin GVIA, which was the first toxin of the series to be chemically characterized.

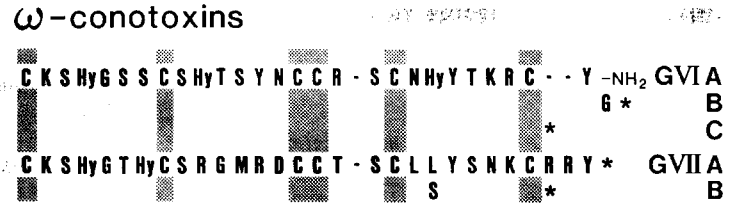

Figure 1. Primary sequences of five ω-conotoxins purified from Conus geographus venom. Asterisks signify presumed blocked C-termini. Most experiments were done using ^{125}I-labeled ω-conotoxin GVIA, modified at Tyr-22.

ω-Conotoxins and Their Targets: Neuronal Voltage Sensitive Ca Channels. A large body of experimental evidence has been obtained which shows that certain voltage sensitive Ca channels are the ω-conotoxin target. Initial electrophysiological data showed a presynaptic block of frog neuromuscular junction, consistent with inhibition of voltage sensitive Ca channels (Kerr and Yoshikami 1984). More recent electrophysiological data have been obtained which definitively demonstrate direct effects on Ca channels (D. Feldman et al., manuscript in preparation). The most elegant data of all are single Ca channel, patch clamp recordings (E. McCleskey et al., manuscript in preparation).

In addition to standard electrophysiological techniques, the direct observation of calcium currents in vitro using synaptosomes has been used for studying ω-conotoxin effects. In these studies as well, ω-conotoxin is able to block voltage sensitive ^{45}Ca-uptake (Reynolds et al. 1986; Rivier et al. 1987). Experiments have been performed in both chick brain and rat brain synaptosomes. Thus, a wide variety of experimental data support the conclusion that the ω-conotoxin target is the voltage-sensitive Ca channel.

Recently, we obtained direct biochemical evidence that the high affinity ω-conotoxin binding site is restricted to neuronal tissue. There are particularly well characterized Ca channels in the T

tubules of skeletal muscle, as well as in cardiac tissue. However, using iodinated ω-conotoxin, we were unable to find high affinity binding sites in these tissues (Cruz et al. 1987). Electrophysiological evidence also indicated no effect of low concentrations of ω-conotoxin on voltage sensitive calcium uptake in these tissues.

In contrast, however, high affinity binding sites were readily demonstrable in a variety of neuronal tissues from several vertebrate sources. ω-Conotoxin blocks Ca channels in a wide variety of neuronal cell types, as determined by electrophysiological methods. Cross-linking data have revealed a denatured MW of 135,000 for the target from chick brain (Cruz et al. 1987). All of these data are consistent with the hypothesis that the ω-conotoxin targets are the large (α) subunits of voltage sensitive Ca channels in neurons.

We recently obtained evidence (to be described elsewhere) which indicates that the targets of ω-conotoxin GVIA are biochemically heterogeneous (G. LeCheminant and D. Griffin, unpublished results). The work from the laboratory of R. Tsien has indicated that the ω-conotoxin target in at least some neuronal tissues is also electrophysiologically heterogeneous (E. McClesky et al., manuscript in preparation). The picture that emerges is that ω-conotoxins can bind and inhibit two types of electrophysiologically distinct Ca channel classes, the so-called L and N type Ca channels. It has not yet been established whether the biochemical heterogeneity corresponds to the electrophysiological classes of voltage-sensitive Ca channels.

ω-Conotoxin: Binding Interactions. Preliminary experiments have been carried out to examine whether drugs and other agents which might affect Ca channel function influence the degree of ω-conotoxin binding. Such effects, whether direct or indirect, could conceivably reveal interactions between the components of the Ca channel complex.

Among the most prominent class of compounds that have been used to study Ca channels are the dihydropyridines. These have proven to be clinically useful as drugs. Dihydropyridines can act either as agonists or antagonists of Ca channels. Nitrendipine, nifedipine PN200-110 and Bay K-600 are among the most commonly used drugs to study Ca channels.

We had previously shown that ω-conotoxin did not compete for the binding with the dihydropyridine drug nitrendipine for sites in skeletal muscle T tubules (Cruz and Olivera 1986). However, since we recently established that ω-conotoxin does not have high affinity targets in non-neuronal tissue, the lack of competition may simply be due to the narrower tissue selectivity of ω-conotoxin compared to the dihydropyridines. Thus, it is desirable to examine binding competition between the dihydropyridines and ω-conotoxin in neuronal tissue. Our previous preliminary results indicated no competition between dihydropyridines and ω-conotoxin even for the neuronal sites. The addition of a large excess of dihydropyridine did not affect ω-conotoxin binding to chick brain receptor sites (Cruz and Olivera 1986).

The converse is also true; ω-conotoxin does not displace the binding of nitrendipine to chick brain synaptosomal sites. Indeed, when ω-conotoxin is added, an increase in dihydropyridine binding is sometimes observed (Table 1) at higher dihydropyridine concentration. These preliminary data suggest a possible interaction between the ω-conotoxin binding site and the dihydropyridine binding site in some of the relevant target Ca channels. Such interactions have been proposed for the dihydropyridine drugs previously with regard to their interactions with other organic Ca channel antagonists.

TABLE 1. Effect of ω-conotoxin on dihydropyridine binding to chick brain membranes

^{3}H-Nimodipine incubation conditions	cpm ^{3}H-Nimodipine, Specifically Bound		
	Concentration of ^{3}H-Nimodipine nM	Specific binding, control	Specific binding with ω-CgTx present
0° for 30 min	2.5	6042	5525
	20.0	7993	10,628
37°C for 30 min	2.5	8055	7685
	5.0	8772	10,034

Dihydropyridine binding assays were carried out as previously described (Cruz and Olivera 1986) except that ^{3}H-Nimodipine (2.54×10^5 cpm/nmol) was used instead of nitrendipine. The binding in the presence or absence of 0.5 μM ω-conotoxin GVIA was determined using 0.52 mg of membrane protein in a total volume of 0.2 ml.

We have also examined the effect of guanine nucleotides on both ω-conotoxin and dihydropyridine binding. The initial results are shown in Figure 2 and Table 2. Incubation of chick brain synaptosomes at 0° does not appreciably affect ω-conotoxin binding, even in the presence of guanine nucleotide analogues. Surprisingly, however, the amount of dihydropyridine (^{3}H-PN200-110) binding depends rather strikingly on whether the synaptosome preparation has been incubated in the presence of a GTP or a GDP analog. Thus, when the preparation is incubated in the presence of GTP-γ-S, a significant decrease in the number of dihydropyridine binding sites is observed. In contrast, when the preparation is incubated in the presence of GDP-β-S, or with no guanine nucleotide added, less of a decline is seen. These results provide tentative biochemical evidence for the modulation of Ca channel activity by G proteins; the fact that only dihydropyridine binding and not ω-conotoxin is adversely affected suggests that dihydropyridines may be sensitive to the state of the channel, while ω-conotoxins are relatively insensitive. These results indicate that dihydropyridine binding can potentially be used to study modulatory influences on certain calcium channel subtypes.

TABLE 2. Effect of guanine nucleotide analogs on Ca channel ligand binding

	Specific Binding to Microsomes (cpm)			
	^{3}H-PN200-110			^{125}I-ωCgTx
Time after microsome preparation (hr)	5	28	46	46
Preparation:				
Lysed microsomes, no addition	4244	2754	3263	17947
Lysed microsomes + GDP-β-S	4707	2284	2678	18897
Lysed microsomes, + GTP-γ-S	3625	941	624	23548

Lysed chick brain membrane preparations were incubated at 0° either with no addition or 0.2 mM GDP-β-S or GTP-γ-S as indicated. Membrane preparations and binding assays were carried out as described previously (Cruz and Olivera 1986), except that ^{3}H-PN-200-110 (8.54 x 10^{7} cpm/nmol, final conc. 15 nM) was used as the dihydropyridine ligand instead of nitrendipine.

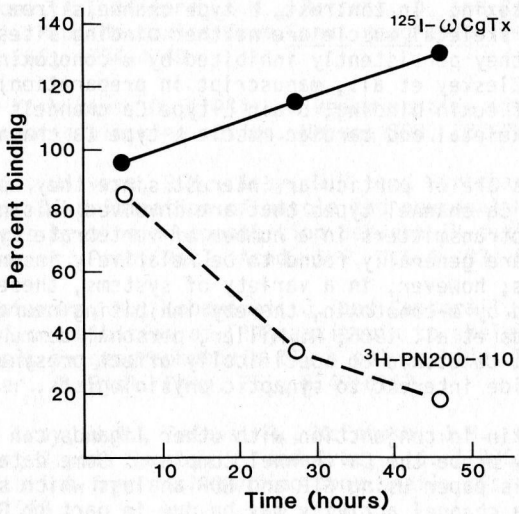

Figure 2. Specific binding assays using ^{125}I-ω-conotoxin and ^{3}H-dihydropyridine were carried out as described for Table 2; the data at each time point are normalized to a control incubation with no guanine nucleotides added.

DISCUSSION

The ω-conotoxins from the venom of the piscivorous marine snails of the genus Conus have been shown to block voltage sensitive Ca channels in neuronal tissue. They thus provide a novel tool for studying Ca channels. We have described our studies on ω-conotoxin GVIA from Conus geographus. Particularly noteworthy is the high affinity and relatively small size of the toxin. The latter property, common to all major classes of conotoxins in C. geographus venom has made it possible to chemically synthesize this and other conotoxins (Olivera et al. 1985). Modified toxins suitable for a variety of applications (i.e., radioactive toxins, toxins with "reporter" groups) have also been made synthetically.

The availability of ω-conotoxins as a probe for Ca channels has revealed the complexity of neuronal Ca channel subtypes, and demonstrated that although electrophysiologically similar channels may be present in neuronal and non-neuronal tissue, the toxin can discriminate between different tissue types. Thus, a slowly inactivating channel sensitive to dihydropyridines has been defined in skeletal muscle, cardiac muscle, and in neuronal tissue. In their general physiological characteristics and in their susceptibility to dihydropyridines, these channels seem to form a reasonably coherent class ("L-type" Ca channels) although minor differences between different tissue types have already been reported. However, the discrimination of ω-conotoxin appears to be almost absolute; the L channels in brain synaptosomes bind ω-conotoxin with relatively high affinity, and are blocked by the toxin. In contrast, L type channels from cardiac tissue and from skeletal muscle are neither binding sites for the toxin, nor are they persistently inhibited by ω-conotoxin (Cruz et al. 1987; E. McCleskey et al., manuscript in preparation). Thus, by the criterion of toxin binding, brain L-type Ca channels appear to be distinct from skeletal and cardiac muscle L-type Ca channels.

The ω-conotoxins are of particular interest since they appear to block the major Ca channel types that are involved in synaptic release of neurotransmitters in a number of vertebrate systems. These channels are generally found to be relatively insensitive to dihydropyridines; however, in a variety of systems, these Ca channels can be inhibited by ω-conotoxin, thereby inhibiting neurotransmitter release (Reynolds et al. 1986; R. Miller, personal communication). The ability of ω-conotoxin to specifically affect presynaptic events is clearly of wide interest to synaptic physiologists.

Finally, the toxin in conjunction with other ligands can be used to more effectively probe the Ca channel complex. Some data have been presented in this paper using GTP and GDP analogs which suggest that modulation of Ca channel activity may be due in part to G proteins. The use of ω-conotoxin with the dihydropyridines in particular should allow a more thorough molecular dissection of neuronal voltage sensitive Ca channels.

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